

# Compositions and Methods for Enhanced Sensitivity and Specificity of Nucleic Acid Synthesis

## 5 *Cross-reference to Related Applications*

This application is related to United States provisional patent application number 60/142,072 filed July 2, 1999, which is specifically incorporated herein in its entirety.

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## *Field of the Invention*

The present invention relates to a method for increasing sensitivity and specificity of nucleic acid synthesis by reducing nonspecific nucleic acid synthesis occurring at ambient temperature. The invention also relates to novel nucleic acids which have high affinity to polymerases. The methods and compositions of the present invention can be used in DNA sequencing, amplification reactions, nucleic acid synthesis and cDNA synthesis.

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The invention also relates to nucleic acids and compositions which are capable of inhibiting or preventing nucleic acid synthesis, sequencing, amplification and cDNA synthesis, for example, by binding one or more polypeptides with polymerase activity. In addition, the materials and methods of the present invention may be used as therapeutics to inhibit the replication of organisms that rely upon a reverse transcriptase activity for completion of their life cycle, such as retroviruses. The invention also relates to vectors and host cells comprising such nucleic acid molecules. The invention also concerns kits comprising the compositions or nucleic acids of the invention.

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## *Background of the Invention*

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DNA polymerases synthesize the formation of DNA molecules which are complementary to a DNA template. Upon hybridization of a primer to the

single-stranded DNA template, polymerases synthesize DNA in the 5' to 3' direction, successively adding nucleotides to the 3'-hydroxyl group of the growing strand. Thus, in the presence of deoxyribonucleoside triphosphates (dNTPs) and a primer, a new DNA molecule, complementary to the single  
5 stranded DNA template, can be synthesized.

Both mesophilic and thermophilic DNA polymerases are used to synthesize nucleic acids. Using thermostable rather than mesophilic polymerases is preferable since the higher annealing temperatures used with thermostable polymerases result in less non-specific DNA amplification from  
10 extension of mis-annealed primers. Even with thermostable polymerases, however, some primer sequences and certain experimental conditions can result in the synthesis of a significant amount of non-specific DNA products. These non-specific products can reduce the sensitivity of polymerase-based  
15 assays and can require extensive optimization for each primer set. In addition, this problem is intensified when polymerases having a high level of activity at ambient temperature are employed (for example, DNA polymerase from *Thermotoga neapolitana*).

In examining the structure and physiology of an organism, tissue or cell, it is often desirable to determine its genetic content. The genetic  
20 framework of an organism is encoded in the double-stranded sequence of nucleotide bases in the deoxyribonucleic acid (DNA) which is contained in the somatic and germ cells of the organism. The genetic content of a particular segment of DNA, or gene, is only manifested upon production of the protein and RNA which the gene encodes. In order to produce a protein, a  
25 complementary copy of one strand of the DNA double helix (the "coding" strand) is produced by polymerase enzymes, resulting in a specific sequence of ribonucleic acid (RNA). This particular type of RNA, since it contains the genetic message from the DNA for production of a protein, is called messenger RNA (mRNA).

30 Within a given cell, tissue or organism, there exist many mRNA species, each encoding a separate and specific protein. This fact provides a

powerful tool to investigators interested in studying genetic expression in a tissue or cell. mRNA molecules may be isolated and further manipulated by various molecular biological techniques, thereby allowing the elucidation of the full functional genetic content of a cell, tissue or organism.

5 A common approach to the study of gene expression is the production of complementary DNA (cDNA) clones. In this technique, the mRNA molecules from an organism are isolated from an extract of the cells or tissues of the organism. This isolation often employs chromatography matrices, such as cellulose or agarose, to which oligomers of thymidine (T) have been  
10 complexed. Since the 3' termini on most eukaryotic mRNA molecules contain a string of Adenosine (A) bases, and since A binds to T, the mRNA molecules can be rapidly purified from other molecules and substances in the tissue or cell extract. From these purified mRNA molecules, cDNA copies may be made using the enzyme reverse transcriptase (RT) or DNA polymerases  
15 having RT activity, which results in the production of single-stranded cDNA molecules. The single-stranded cDNAs may then be converted into a complete double-stranded DNA copy (*i.e.*, a double-stranded cDNA) of the original mRNA (and thus of the original double-stranded DNA sequences, encoding this mRNA, contained in the genome of the organism) by the action of a DNA polymerase. The protein-specific double-stranded cDNAs can then be inserted into a vector, which is then introduced into a host bacterial, yeast, animal or plant cell, a process referred to as transformation or transfection. The host cells are then grown in culture media, resulting in a population of host cells containing (or in many cases, expressing) the gene of interest or  
20 portions of the gene of interest.

25 This entire process, from isolation of mRNA to insertion of the cDNA into a vector (e.g., plasmid, viral vector, cosmid, etc.) to growth of host cell populations containing the isolated gene or gene portions, is termed "cDNA cloning." If cDNAs are prepared from a number of different mRNAs, the  
30 resulting set of cDNAs is called a "cDNA library," an appropriate term since the set of cDNAs represents a "population" of genes or portions of genes

comprising the functional genetic information present in the source cell, tissue or organism.

Synthesis of a cDNA molecule initiates at or near the 3' termini of the mRNA molecules and proceeds in the 5'-to-3' direction successively adding nucleotides to the growing strand. Priming of the cDNA synthesis at the 3'-termini at the poly A tail using an oligo (dT) primer ensures that the 3' message of the mRNAs will be represented in the cDNA molecules produced. The ability to increase sensitivity and specificity during cDNA synthesis provides more representative cDNA libraries and may increase the likelihood of the cDNA library having full-length cDNA molecules (e.g., full-length genes). Such advances would greatly improve the probability of finding full-length genes of interest.

In addition to their importance for research purposes, reverse transcriptase enzymes play a critical role in the life cycle of many important pathogenic viruses, in particular, the human immunodeficiency viruses (HIV). In order to complete its life cycle, HIV and other similar viruses must use a reverse transcriptase enzyme to convert the viral RNA genome into DNA for integration into the host's genomic material. Since this step is critical to the viral life cycle and host cells do not have any similar requirement for reverse transcriptase activity, the reverse transcriptase enzyme has been intensively studied as a chemotherapeutic target. In general, the bulk of therapeutic reagents directed at the reverse transcriptase enzyme have been nucleotide analogues, for example AZT. Other therapeutic modalities using oligonucleotide-based reagents, e.g., anti-sense oligonucleotides and ribozymes, have been used to inhibit viral replication, however, these reagents are not targeted specifically against reverse transcriptase activity, instead of being targeted against the nucleic acid of the viral genome. See, for example, Goodchild, *et al.*, "Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides," *Proc. Natl. Acad. Sci. USA* 85:5507-5511 (1988), Matsukara, *et al.*, "Regulation of viral expression of human immunodeficiency virus in vitro by an antisense phosphorothioate

oligodeoxynucleotide against rev (art/trs) in chronically infected cells," *Proc. Natl. Acad. Sci. USA* 86:4244-4248 (1989), Rossi, *et al.*, "Ribozymes as Anti-HIV-1 Therapeutic Agents: Principles, Applications, and Problems," *Aids Research and Human Retroviruses* 8:183:189 (1992), Goodchild, "Enhancement of ribozyme catalytic activity by a contiguous oligodeoxynucleotide (facilitator) and by 2'-O-methylation," *Nucleic Acids Research* 20:4607-4612 (1992) and Kinchington, *et al.*, "A comparison of gag, pol and rev antisense oligodeoxynucleotides as inhibitors of HIV-1," *Antiviral Research* 17:53-62 (1992) which are specifically incorporated herein by reference. Oligonucleotides that have been blocked at the 3'-end to prevent their elongation by reverse transcriptase have also been considered as inhibitors (see, for example, Austermann, *et al.*, "Inhibition of human immunodeficiency virus type 1 reverse transcriptase by 3'-blocked oligonucleotides" *Biochemical Pharmacology* 43(12):2581-2589 (1992). Each of the above cited references is specifically incorporated herein in its entirety.

Oligonucleotides have been investigated for anti-HIV activity. For example, Idriss, *et al.* (1994), *Journal of Enzyme Inhibition* 8(2)97-112, disclose DNA oligonucleotides in a hairpin structure as inhibitors of HIV RT activity while Kuwasaki, *et al.*, (1996) *Biochemical and Biophysical Research Communications* 228:623-631 disclose anti-sense hairpin oligonucleotides containing a mixture of deoxy and 2'-methoxy-nucleotides with anti-HIV activity.

Notwithstanding these and other efforts to modulate the activity of polymerases, there remains a need in the art for materials and methods to prevent the undesirable activity of the polymerases while permitting the synthesis of nucleic acids by the polymerase when such synthesis is desired. These and other needs are met by the present invention.

*Summary of the Invention*

5 The present invention provides materials and methods for inhibiting, reducing, substantially reducing or eliminating nucleic acid synthesis under certain conditions (preferably at ambient temperatures and/or within a cell) while permitting synthesis when such synthesis is desired.

10 In a preferred aspect, the invention relates to methods for the prevention or inhibition of nucleic acid synthesis during reaction set up (e.g., in vitro) and preferably before optimum reaction conditions for nucleic acid synthesis are achieved. Such inhibition of synthesis at sub-optimum conditions or during reaction set up prevents or reduces non-specific nucleic acid synthesis. Once reaction set up is complete and the optimum conditions are reached, nucleic acid synthesis can be initiated.

15 In another aspect, the present invention relates to a method of inhibiting a polymerase enzyme within a cell (e.g., in vivo) by introducing into the cell an oligonucleotide or inhibitor of the invention, preferably said oligonucleotide comprises a 5'-and a 3'-portion, wherein the said 3'-portion comprises one or more deoxyribonucleotides or derivatives thereof and said 5'-portion comprises one or more ribonucleotides or derivatives thereof and  
20 wherein all or a portion of said 3'-portion is capable of base pairing to all or a portion of said 5'-portion and incubating said cell under conditions causing the inhibition of the polymerase. In some embodiments, the 5'-portion of the oligonucleotide which comprises ribonucleotides forms a 5'-overhang. In another aspect, the oligonucleotide is in the form of a hairpin and preferably  
25 the stem of the hairpin comprises a series of contiguous ribonucleotides based paired or hybridized with a series of contiguous deoxyribonucleotides. In some embodiments the polymerase is a reverse transcriptase and may preferably be an HIV reverse transcriptase.

30 In another aspect, the present invention provides a method of inhibiting replication of a virus, by providing a virus, said virus comprising a reverse transcriptase and requiring activity of the reverse transcriptase for replication



complexed with other molecules such as peptides or proteins and the like. Such inhibitory nucleic acids preferably include double-stranded nucleic acid molecules (which may comprise one or more internal, 5' and/or 3' single stranded portions), or single stranded nucleic acid molecules capable of  
5 folding into a double stranded form, i.e. forming one or more hairpin-loops, such that at least one double stranded portion of the nucleic acid molecule is capable of binding to a polypeptide with polymerase activity. In one aspect, the nucleic acid molecules used in the invention bind the polypeptide having polymerase activity (e.g., DNA polymerase, reverse transcriptase, etc.) with  
10 high affinity. Once the polymerase or reverse transcriptase is complexed with the inhibitory nucleic acid, it is unavailable for annealing to the primer/template substrate, resulting in reduced, substantially reduced, or no polymerase or reverse transcriptase activity. In some embodiments, the oligonucleotides of the present invention may comprise a 5'- and a 3'-portion,  
15 wherein said 3'-portion comprises one or more deoxyribonucleotides or derivatives thereof and said 5'-portion comprises one or more ribonucleotides or derivatives thereof and wherein all or a portion of said 3'-portion is capable of base pairing to all or a portion of said 5'-portion. In some embodiments, the oligonucleotides of the invention may comprise a 5'-portion, wherein said  
20 5'-portion comprising ribonucleotides forms a 5'-overhang. In some embodiments, an oligonucleotide of the invention may comprise one or more modifications so as to be non-extendable. In some embodiments, this modification may be to the 3'-most nucleotide. In some embodiments, the modification is phosphorylation of the 3'-most nucleotide at the 3'-hydroxyl.  
25 An oligonucleotide of the present invention may comprise one or more modifications so as to be resistant to digestion or degradation by, for example, one or more nucleases. In some embodiments, this modification may be the incorporation of one or more phosphorothioate moieties. In some embodiments, the modification may comprise alkylation of one or more  
30 hydroxyl groups.



Thus, the inhibitory nucleic acid is preferably introduced into the reaction mixture where it competitively binds to or interacts with the polymerase, thereby inhibiting synthesis by the polymerase under particular reaction conditions. Thus, interaction or binding of the inhibitor and polymerase preferably results in the formation of an inhibitor/polymerase complex.

The inhibition of polymerase activity or nucleic acid synthesis by the nucleic acids of the invention is preferably reduced, substantially reduced, inhibited, or eliminated so that nucleic acid synthesis may proceed when reaction conditions are changed, for example, when the temperature is raised. In a preferred aspect, the changed conditions affect the ability of the inhibitory nucleic acids to interact with the polymerase causing release of the polymerase and/or denaturation or inactivation of the inhibitory nucleic acids making the polymerase available thus allowing nucleic acid synthesis to proceed. In one aspect, the inhibitory nucleic acids and the primer/template substrate competitively interact with the polymerase to prevent synthesis. Under the changed conditions, the competitive interaction is reduced such that nucleic acid synthesis occurs. In another aspect, the changed conditions cause the double stranded inhibitory nucleic acid molecule(s) (including hairpins) to denature or melt such that single stranded molecules are formed which do not substantially bind or interact with the polymerase. In another aspect, a second change in conditions (i.e., temperature is lowered to, for example, ambient temperatures) allows the inhibitor nucleic acid molecules of the invention to reactivate or again inhibit nucleic acid synthesis. That is, the inhibitors may again interact or bind with the polymerase or reverse transcriptase under the changed conditions. For example, the changed conditions may allow the inhibitor to form double stranded molecules which effectively enhances its binding or interacting capacity with the polymerase or reverse transcriptases. Thus, in accordance with the invention, the inhibitors may be reused or recycleable during synthesis reactions (single or multiple) which may require



templates with one or more primers, and one or more inhibitory nucleic acid molecules or compositions of the present invention capable of binding or interacting with an enzyme having polymerase activity and (b) incubating the mixture in the presence of one or more enzymes having nucleic acid polymerase activity (e.g., DNA polymerases) under conditions sufficient to amplify one or more nucleic acid molecules complementary to all or a portion of the templates. More specifically, the invention relates to a method of amplifying a DNA molecule comprising: (a) providing a first and second primer, wherein said first primer is complementary to a sequence within or at or near the 3'-termini of the first strand of said DNA molecule and said second primer is complementary to a sequence within or at or near the 3'-termini of the second strand of said DNA molecule, and one or more inhibitory nucleic acids or compositions of the invention (e.g., a nucleic acid having affinity for an enzyme with polymerase activity); (b) hybridizing said first primer to said first strand and said second primer to said second strand; (c) incubating the mixture under conditions such that a third DNA molecule complementary to all or a portion of said first strand and a fourth DNA molecule complementary to all or a portion of said second strand are synthesized; (d) denaturing said first and third strand, and said second and fourth strands; and (e) repeating steps (a) to (c) or (d) one or more times. Such conditions may include incubation in the presence of one or more polymerases, one or more nucleotides and/or one or more buffering salts. The invention also relates to nucleic acid molecules amplified by these methods.

In a related aspect, the nucleic acid amplification method may comprise (a) mixing one or more polymerases with one or more of the inhibitory nucleic acid molecules of the invention, and (b) incubating such mixture under conditions sufficient to inactivate or substantially inhibit or reduce polymerase activity of such polymerases. In another aspect, such incubation is under conditions sufficient to inhibit or prevent such nucleic acid amplification.

The invention also relates to methods for sequencing a nucleic acid molecule comprising (a) mixing a nucleic acid molecule to be sequenced with one or more primers, one or more of the inhibitory nucleic acids or compositions of the invention, one or more nucleotides and one or more terminating agents to form a mixture; (b) incubating the mixture under conditions sufficient to synthesize a population of molecules complementary to all or a portion of the molecule to be sequenced; and (c) separating the population to determine the nucleotide sequences of all or a portion of the molecule to be sequenced. The invention more specifically relates to a method of sequencing a nucleic acid molecule, comprising: (a) providing an inhibitory nucleic acid or composition of the present invention (to which an enzyme with polymerase activity as affinity), one or more nucleotides, and one or more terminating agents; (b) hybridizing a primer to a first nucleic acid molecule; (c) incubating the mixture of step (b) under conditions sufficient to synthesize a random population of nucleic acid molecules complementary to said first nucleic acid molecule, wherein said synthesized molecules are shorter in length than said first molecule and wherein said synthesized molecules comprise a terminator nucleotide at their 3' termini; and (d) separating said synthesized molecules by size so that at least a part of the nucleotide sequences of said first nucleic acid molecule can be determined. Such terminator nucleotides include dideoxynucleoside triphosphates such as ddNTP, ddATP, ddGTP, ddITP or ddCTP. Such conditions may include incubation in the presence of one or more polymerases and/or buffering salts.

In a related aspect, the nucleic acid sequencing method may comprise (a) mixing one or more polymerases with one or more of the inhibitory nucleic acid molecules of the invention, and (b) incubating such mixture under conditions sufficient to inactivate or substantially inhibit polymerase activity of such polymerases. In another aspect, such incubation is under conditions sufficient to inhibit or prevent such nucleic acid sequencing.

The invention also relates to the inhibitory nucleic acids of the invention and to compositions comprising the inhibitory nucleic acids of the



by well known techniques. In some embodiments, the present invention provides a method of inhibiting the growth of a virus, comprising contacting a cell infected with a virus that requires reverse transcriptase activity to complete its life cycle with an inhibitory oligonucleotide under conditions  
5 causing the oligonucleotide to be taken up by the cell and causing the reverse transcriptase to be inhibited thereby inhibiting the growth of the virus. In some embodiments, the present invention provides a method of treating an organism or subject infected with a virus that requires reverse transcriptase activity to complete its life cycle comprising contacting an infected cell of the  
10 organism or subject with a composition comprising an inhibitory oligonucleotide under conditions causing the oligonucleotide to be taken up by the cell and causing the reverse transcriptase to be inhibited thereby treating the organism.

The invention also relates to kits for use in synthesis, sequencing and  
15 amplification of nucleic acid molecules, comprising one or more containers containing one or more of the inhibitory nucleic acids or compositions of the invention. These kits of the invention may optionally comprise one or more additional components selected from the group consisting of one or more nucleotides, one or more polymerases (e.g., thermophilic or mesophilic DNA  
20 polymerases and/or reverse transcriptases), one or more suitable buffers, one or more primers and one or more terminating agents (such as one or more dideoxynucleotides). The invention also relates to kits for inhibiting viral replication or kits for treating viral infections comprising the inhibitory nucleic acids of the invention. Such kits may also comprise instructions or protocols  
25 for carrying out the methods of the invention.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of the following drawings and description of the invention, and of the claims.

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### ***Brief Description of the Drawings***

**Figure 1.** The activity of *Tne* DNA polymerase was qualitatively determined. Panel A: determination in the absence of inhibitor A, Panel B: determination in the presence of inhibitor. The five lanes, of each panel, from left to right are 15 sec, 30 sec, 1 min, 2 min and 5 min time points that have elapsed before the reactions were quenched. P and C denote the primer position and control lane, respectively. Inhibitor A did not contain dideoxynucleotide at its termini.

**Figure 2.** Amplification of a 2.7 Kb target DNA sequence (pUC19) was performed at 5 different dilutions of the template. The target was amplified by *Tne* DNA polymerase. Two different concentration of *Tne* polymerase (85 nM and (e.g, 1 unit) and 42.5 nM (e.g., 0.5 units)) and the respective inhibitor B Complexes (using a 150-fold excess hairpin B over the polymerase concentration) were used for each amplification condition. The concentration of the target DNA in lanes 1, 2, 3, 4 & 5 is 100 pg, 20 pg, 2 pg, 0.2 pg and 0.02 pg, respectively. Inhibitor B did not contain dideoxynucleotide at its termini.

**Figure 3.** A 1Kb, 3 Kb and 5Kb target DNA sequence (human genomic source) were amplified by *Tne* (85 nM (e.g., 1 unit) and *Taq* (e.g., 1 unit)) DNA polymerases as represented in panels A, B, and C respectively. The four lanes of each panel represented as a, b, c and d are *Tne* (+ 125-fold excess inhibitor A), *Tne* (+50-fold excess inhibitor A), *Tne* (no inhibitor) and *Taq* (no inhibitor), respectively. Inhibitor A contained a 3'-terminal dideoxynucleotide (i.e., ddT).

**Figure 4.** The amplification of a 5 and 15 Kb target DNA sequence (human genomic source) was performed with *Tne* DNA polymerase (8.5 nM (e.g., 0.1 units)). The five panels A, B, C, D and E represent reaction conditions: *Tne* (no inhibitor), *Tne* (+ 50-fold excess inhibitor), *Tne* (+150-fold excess inhibitor), *Tne* (+300-fold excess inhibitor), *Tne* (+750-fold excess inhibitor), respectively. The two lanes (a and b) for each panel represent

amplification of a target size of 5 and 15 Kb. For this assay, the inhibitor B was used which did not contain a terminal dideoxynucleotide.

5 **Figure 5.** The amplification of a 3 Kb target DNA sequence (human genomic source) was performed with 1 unit *Taq* DNA polymerase. The three panels A, B, and C represent reaction conditions: for A and B the same primer sequences were used – I) the PCR mix was incubated at 94°C for 1 min and was set on ice to force mis-priming. All PCR reactions were set for 30 min at 25°C so as to increase non-specific DNA synthesis. Each condition has four lanes-lane a (*Taq* control), b (*Taq* + 16 nM inhibitor), c (*Taq* + 32 nM inhibitor) and d (*Taq* + 64 nM inhibitor). Inhibitor B was used which did not contain a terminal dideoxynucleotide.

10 **Figure 6A** is a graph showing the results of polymerase activity assays of Thermoscript™ I in the absence and presence of nucleic acid inhibitors at ambient temperature (left bar), 37°C (center bar) and 55°C (right bar). TS denotes polymerase reaction initiated by Thermoscript™ I, TS-D denotes polymerase reaction initiated by Thermoscript™ I in the presence of nucleic acid inhibitor D, TS-E denotes polymerase reaction initiated by Thermoscript™ I in the presence of nucleic acid inhibitor E, and TS-H denotes polymerase reaction initiated by Thermoscript™ I in the presence of nucleic acid inhibitor H.

15 **Figure 6B** is a graph showing the results of polymerase activity assays of Thermoscript™ I in the absence and presence of nucleic acid inhibitors at ambient temperature (left bar), 37°C (center bar) and 55°C (right bar). TS denotes polymerase reaction initiated by Thermoscript™ I, TS-C denotes polymerase reaction initiated by Thermoscript™ I in the presence of nucleic acid inhibitor C, TS-H denotes polymerase reaction initiated by Thermoscript™ I in the presence of nucleic acid inhibitor H, TS-E denotes polymerase reaction initiated by Thermoscript™ I in the presence of nucleic acid inhibitor E, TS-F denotes polymerase reaction initiated by Thermoscript™ I in the presence of nucleic acid inhibitor F, and TS-G denotes

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polymerase reaction initiated by Thermoscript™ I in the presence of nucleic acid inhibitor G.

5 **Figure 7** is a photograph of an agarose gel showing the results of amplification reactions using a 1.6 kb (A), a 2 kb (B) and a 2.6 kb (C) fragment of the NF2 gene. In each panel, lane a is the amplification using *Taq* polymerase alone, lane b is the amplification reaction in the presence of inhibitor HPHH4Sspa3 at a molar ratio of 1.2:1 inhibitor: polymerase and lane c is the amplification using Platinum *Taq*.

10 **Figure 8** is a bar graph showing the results of a dNTP incorporation assay at the indicated temperatures. At each temperature, the solid black rectangle reports the results obtained with *Taq* polymerase alone, the striped rectangle report the results obtained with inhibitor HPHH4Sspa3 at a molar ratio of 2:1 inhibitor:polymerase, the white rectangle report the results obtained with the same inhibitor at a molar ratio of 7.5:1 inhibitor:polymerase.

15 **Figure 9** is a graph showing the dose dependent inhibition of *Taq* polymerase by inhibitor present HPHH4Sspa3 present at the indicated molar ratios.

## 20 *Detailed Description of the Invention*

### *Definitions*

25 In the description that follows, a number of terms used in recombinant DNA technology are utilized extensively. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

**Primer.** As used herein, “primer” refers to a single-stranded oligonucleotide that is extended by covalent bonding of nucleotide monomers during amplification or polymerization of a DNA molecule.

30 **Template.** The term “template” as used herein refers to double-stranded or single-stranded nucleic acid molecules which are to be amplified,

synthesized or sequenced. In the case of a double-stranded molecule, denaturation of its strands to form a first and second strand is preferably performed before these molecules may be amplified, synthesized or sequenced, or the double stranded molecule may be used directly as a template. For single stranded templates, a primer, complementary to a portion of the template is hybridized under appropriate conditions and one or more polymerases may then synthesize a nucleic acid molecule complementary to all or a portion of said template. Alternatively, for double stranded templates, one or more promoters (e.g. SP6, T7 or T3 promoters) may be used in combination with one or more polymerases to make nucleic acid molecules complementary to all or a portion of the template. The newly synthesized molecules, according to the invention, may be equal or shorter in length than the original template.

***Incorporating.*** The term “incorporating” as used herein means becoming a part of a DNA and/or RNA molecule or primer.

***Amplification.*** As used herein “amplification” refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence with the use of a polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a DNA and/or RNA molecule or primer thereby forming a new molecule complementary to a template. The formed nucleic acid molecule and its template can be used as templates to synthesize additional nucleic acid molecules. As used herein, one amplification reaction may consist of many rounds of replication. DNA amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 5 to 100 “cycles” of denaturation and synthesis of a DNA molecule.

***Nucleotide.*** As used herein “nucleotide” refers to a base-sugar-phosphate combination. Nucleotides are monomeric units of a nucleic acid sequence (DNA and RNA). Nucleotides may also include mono-, di- and triphosphate forms of such nucleotides. The term nucleotide includes ribonucleoside triphosphates ATP, UTP, CTG, GTP and deoxyribonucleoside triphosphates such as dATP, dCTP, dITP, dUTP, dGTP, dTTP, or derivatives

thereof. Such derivatives include, for example, [ $\alpha$ S]dATP, 7-deaza-dGTP and 7-deaza-dATP, and nucleotide derivatives that confer nuclease resistance on the nucleic acid molecule containing them. The term nucleotide as used herein also refers to dideoxynucleoside triphosphates (ddNTPs) and their derivatives. Illustrated examples of dideoxynucleoside triphosphates include, but are not limited to, ddATP, ddCTP, ddGTP, ddITP, and ddTTP. According to the present invention, a "nucleotide" may be unlabeled or detectably labeled by well known techniques. Detectable labels include, for example, radioactive isotopes, fluorescent labels, chemiluminescent labels, bioluminescent labels and enzyme labels.

**Blocking Agent.** "Blocking agent" refers to a nucleotide (or derivatives thereof), modified oligonucleotides and/or one or more other modifications which are incorporated into the nucleic acid inhibitors of the invention to prevent or inhibit degradation or digestion of such nucleic acid molecules by nuclease activity. One or multiple blocking agents may be incorporated in the nucleic acid inhibitors of the invention internally, at or near the 3' termini and/or at or near the 5' termini of the nucleic acid inhibitors. Preferably, such blocking agents are located, for linear inhibitor nucleic acid molecules, at or near the 3' termini and/or at or near the 5' termini and/or at the preferred cleavage position of the 5' to 3' exonuclease of such molecules (Lyamichev, V., Brow, M.A.D., and Dahlberg, J.E., (1993) *Science*, 260, 778-783). Preferably, such blocking agents prevent or inhibit degradation or digestion of the inhibitor nucleic acid molecules by exonuclease activity associated with the polymerase or reverse transcriptase used or that may be present in the synthesis reaction. For example, blocking agents for the invention prevent degradation or digestion of inhibitor nucleic acid molecules by 3' exonuclease activity and/or 5' exonuclease activity associated with a polymerase (e.g., a DNA polymerase). Preferred blocking agents in accordance with the invention include dideoxynucleotides and their derivatives such as ddATP, ddCTP, ddGTP, ddITP, and ddTTP. Other blocking agents for use in accordance with the invention include, but are not limited to, AZT,



*Hairpin.* As used herein, the term "hairpin" is used to indicate the structure of an oligonucleotide in which one or more portions of the oligonucleotide form base pairs with one or more other portions of the oligonucleotide. When the two portions are base paired to form a double stranded portion of the oligonucleotide, the double stranded portion may be referred to as a stem. Thus, depending on the number of complementary portions used, a number of stems (preferably 1-10) may be formed. Additionally, formation of the one or more stems preferably allows formation of one or more loop structures in the hairpin molecule. In one aspect, any one or more of the loop structures may be cut or nicked at one or more sites within the loop or loops but preferably at least one loop is not so cut or nicked. The sequence of the oligonucleotide may be selected so as to vary the number of nucleotides which base pair to form the stem from about 3 nucleotides to about 100 or more nucleotides, from about 3 nucleotides to about 50 nucleotides, from about 3 nucleotides to about 25 nucleotides, and from about 3 to about 10 nucleotides. In addition, the sequence of the oligonucleotide may be varied so as to vary the number of nucleotides which do not form base pairs from 0 nucleotides to about 100 or more nucleotides, from 0 nucleotides to about 50 nucleotides, from 0 nucleotides to about 25 nucleotides or from 0 to about 10 nucleotides. The two portions of the oligonucleotide which base pair may be located anywhere or at any number of locations in the sequence of the oligonucleotide. In some embodiments, one base-pairing-portion of the oligonucleotide may include the 3'-terminal of the oligonucleotide. In some embodiments, one base-pairing-portion may include the 5'-terminal of the oligonucleotide. In some embodiments, one base-pairing-portion of the oligonucleotide may include the 3'-terminal while the other base-pairing-portion may include the 5'-terminal and, when base paired, the stem of the oligonucleotide is blunt ended. In other embodiments, the location of the base pairing portions of the oligonucleotide may be selected so as to form a 3'-overhang, a 5'-overhang and/or may be selected so that neither the 3'- nor the 5'-most nucleotides are involved in base pairing.

*Hybridization.* The terms "hybridization" and "hybridizing" refer to base pairing of two complementary single-stranded nucleic acid molecules (RNA and/or DNA and/or PNA) to give a double-stranded molecule. As used herein, two nucleic acid molecules may be hybridized although the base pairing is not completely complementary. Accordingly, mismatched bases do not prevent hybridization of two nucleic acid molecules provided that appropriate conditions, well known in the art, are used. In a preferred aspect, the double stranded inhibitory molecules are denatured under certain conditions such that the complementary single stranded molecules which are hybridized are allowed to separate. Single stranded molecules formed do not interact or bind polymerase or interact or bind polymerase with reduced efficiency compared to the corresponding double-stranded molecule.

*Unit.* The term "unit" as used herein refers to the activity of an enzyme. When referring, for example, to a DNA polymerase, one unit of activity is the amount of enzyme that will incorporate 10 nanomoles of dNTPs into acid-insoluble material (i.e., DNA or RNA) in 30 minutes under standard primed DNA synthesis conditions.

*Viruses.* As used herein, viruses that require a reverse transcriptase activity to complete their lifecycle are seen to include, but are not limited to, any member of the family retroviridae including human immunodeficiency viruses, bovine immunodeficiency virus, bovine leukemia virus, human T-lymphotrophic viruses, caprine arthritis-encephalitis virus, equine infectious anemia virus, feline immunodeficiency virus, feline sarcoma and leukemia viruses, maedi/visna virus of sheep, mouse mammary tumor virus, simian immunodeficiency virus and other retroviruses known to those skilled in the art.

*Vector.* The term "vector" as used herein refers to a plasmid, phagemid, cosmid or phage nucleic acid or other nucleic acid molecule which is able to replicate autonomously in a host cell. Preferably a vector is characterized by one or a small number of restriction endonuclease recognition sites at which such nucleic acid sequences may be cut in a determinable

fashion without loss of an essential biological function of the vector, and into which nucleic acid molecules may be spliced in order to bring about its replication and cloning. The cloning vector may further contain one or more markers suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, are antibiotic resistance genes, including, but not limited to tetracycline resistance or ampicillin resistance.

**Expression vector.** The term "expression vector" as used herein refers to a vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences.

**Recombinant host.** The term "recombinant host" as used herein refers to any prokaryotic or eukaryotic microorganism which contains the desired cloned genes in an expression vector, cloning vector or any other nucleic acid molecule. The term "recombinant host" is also meant to include those host cells which have been genetically engineered to contain the desired gene on a host chromosome or in the host genome.

**Host.** The term "host" as used herein refers to any prokaryotic or eukaryotic microorganism that is the recipient of a replicable expression vector, cloning vector or any nucleic acid molecule including the inhibitory nucleic acid molecules of the invention. The nucleic acid molecule may contain, but is not limited to, a structural gene, a promoter and/or an origin of replication.

**Promoter.** The term "promoter" as used herein refers to a DNA sequence generally described as the 5' region of a gene, located proximal to start the codon. At the promoter region, transcription of an adjacent gene(s) is initiated.

**Gene.** The term "gene" as used herein refers to a DNA sequence that contains information necessary for expression of a polypeptide or protein. It includes the promoter and the structural gene as well as other sequences involved in expression of the protein.

**Structural gene.** The term "structural gene" as used herein refers to a DNA sequence that is transcribed into messenger RNA that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

5 **Operably linked.** The term "operably linked" as used herein means that the promoter is positioned to control the initiation of expression of the polypeptide encoded by the structural gene.

10 **Expression.** The term "expression" as used herein refers to the process by which a gene produces a polypeptide. It includes transcription of the gene into messenger RNA (mRNA) and the translation of such mRNA into polypeptide(s).

15 **Substantially Pure.** As used herein "substantially pure" means that the desired purified molecule such as a protein or nucleic acid molecule (including the inhibitory nucleic acid molecule of the invention) is essentially free from contaminants which are typically associated with the desired molecule. Contaminating components may include, but are not limited to, compounds or molecules which may interfere with the inhibitory or synthesis reactions of the invention, and/or that degrade or digest the inhibitory nucleic acid molecules of the invention (such as nucleases including exonucleases and endonucleases) or that degrade or digest the synthesized or amplified nucleic acid molecules produced by the methods of the invention.

20 **Thermostable.** As used herein "thermostable" refers to a DNA polymerase which is more resistant to inactivation by heat. DNA polymerases synthesize the formation of a DNA molecule complementary to a single-stranded DNA template by extending a primer in the 5'-3'-direction. This activity for mesophilic DNA polymerases may be inactivated by heat treatment. For example, T5 DNA polymerase activity is totally inactivated by exposing the enzyme to a temperature of 90°C for 30 seconds. As used herein, a thermostable DNA polymerase activity is more resistant to heat inactivation than a mesophilic DNA polymerase. However, a thermostable DNA polymerase does not mean to refer to an enzyme which is totally resistant to heat inactivation and thus heat treatment may reduce the DNA polymerase

25  
30



activity to some extent. A thermostable DNA polymerase typically will also have a higher optimum temperature than mesophilic DNA polymerases.

**3'-to-5' Exonuclease Activity.** "3'-to-5' exonuclease activity" is an enzymatic activity well known to the art. This activity is often associated with DNA polymerases and is thought to be involved in a DNA replication "editing" or correction mechanism.

A "DNA polymerase substantially reduced in 3'-to-5' exonuclease activity" is defined herein as either (1) a mutated DNA polymerase that has about or less than 10%, or preferably about or less than 1%, of the 3'-to-5' exonuclease activity of the corresponding unmutated, wild-type enzyme, or (2) a DNA polymerase having a 3'-to-5' exonuclease specific activity which is less than about 1 unit/mg protein, or preferably about or less than 0.1 units/mg protein. A unit of activity of 3'-to-5' exonuclease is defined as the amount of activity that solubilizes 10 nmoles of substrate ends in 60 min. at 37°C, assayed as described in the "BRL 1989 Catalogue & Reference Guide", page 5, with HhaI fragments of lambda DNA 3'-end labeled with [<sup>3</sup>H]dTTP by terminal deoxynucleotidyl transferase (TdT). Protein is measured by the method of Brandford, Anal. Biochem. 72:248 (1976). As a means of comparison, natural, wild-type T5-DNA polymerase (DNAP) or T5-DNAP encoded by pTTQ19-T5-2 has a specific activity of about 10 units/mg protein while the DNA polymerase encoded by pTTQ19-T5-2(Exo-) (U.S. 5,270,179) has a specific activity of about 0.0001 units/mg protein, or 0.001% of the specific activity of the unmodified enzyme, a 10<sup>5</sup>-fold reduction. Polymerases used in accordance with the invention may lack or may be substantially reduced in 3' exonuclease activity.

**5'-to-3' Exonuclease Activity.** "5'-to-3' exonuclease activity" is also enzymatic activity well known in the art. This activity is often associated with DNA polymerases, such as *E. coli PolI* and *Taq* DNA polymerase.

A "polymerase substantially reduced in 5'-to-3' exonuclease activity" is defined herein as either (1) mutated or modified polymerase that has about or less than 10%, or preferably about or less than 1%, of the 5'-to-3'

exonuclease activity of the corresponding unmutated, wild-type enzyme, or (2) a polymerase having 5'-to-3' exonuclease specific activity which is less than about 1 unit/mg protein, or preferably about or less than 0.1 units/mg protein.

Both of the 3'-to-5' and 5'-to-3' exonuclease activities can be observed on sequencing gels. Active 5'-to-3' exonuclease activity will produce different size products in a sequencing gel by removing mono-nucleotides and longer products from the 5'-end of the growing primers. 3'-to-5' exonuclease activity can be measured by following the degradation of radiolabeled primers in a sequencing gel. Thus, the relative amounts of these activities (e.g., by comparing wild-type and mutant or modified polymerases) can be determined with no more than routine experimentation.

*Inhibitory nucleic acids.* The nucleic acids of the present invention include single stranded and double stranded nucleic acids (although other strand multiples such as triple stranded (e.g., triple helix) molecules may be used) including nucleic acids comprised of DNA, RNA, PNA, LNA or other derivative nucleic acid molecules, or a combination thereof. The inhibitory nucleic acid comprises a sequence which is capable of forming a site at one set of conditions (preferably at ambient temperature) which competes with the template/primer substrate used in synthesis or amplification for binding an enzyme with polymerase activity and competes less efficiently under a second set of conditions (preferably elevated temperatures) for nucleic acid synthesis or amplification. Preferably, the sequence of the inhibitory nucleic acids is not complementary to the primer used in the synthesis, amplification or sequencing reaction to be inhibited. As will be recognized, other nucleic acids (natural, unnatural, modified etc.) may be selected and used in accordance with the invention. Such selection may be accomplished by binding studies and/or nucleic acid synthesis inhibition assays. Design of nucleic acid sequences for hairpin formation may be accomplished by those skilled in the art. See, e.g., Antao, V.P. and Tinoco, I., Jr., 1992, *Nucl. Acids Res.* 20: 819-824. Preferably, the nucleic acid inhibitor could be made nuclease resistant (3'-to-5' exonuclease and 5'-to-3' exonuclease) and/or inert to polymerization.

Methods to render the nucleic acid inert to exonucleases and polymerization are known in the art and include, for example, using derivative nucleic acid molecules which may include derivative nucleotides (for example, using phosphamide and/or phosphorothioate backbone rather than phosphate) and/or addition of one or more blocking agents to the inhibitory nucleic acid molecules of the invention. The inhibitory nucleic acid preferably form one or more hairpin-loop structures with a double stranded stem. The double stranded stem can have blunt ends and/or a single stranded overhang (for example, at the 5' and/or 3' terminus) designed so as to mimic the typical primer/template substrate of a polymerase.

Inhibitory nucleic acids of the present invention are preferably used in the present compositions and methods at a final concentration in a synthesis, sequencing or amplification reaction sufficient to prevent or inhibit such synthesis, sequencing or amplification in the presence of a polymerase or reverse transcriptase enzyme. The ratio of inhibitory nucleic acids of the invention to polymerase or reverse transcriptase may vary depending on the polymerase or reverse transcriptase used. The molar ratio of inhibitory nucleic acids to polymerase/reverse transcriptase enzyme for a synthesis, sequencing or amplification reaction may range from about 0.001 – 100:1; 0.01 – 1000:1; 0.1 – 10,000:1; 1 – 100,000:1; 1 – 500,000:1; or 1 – 1,000,000:1. Of course, other suitable ratios of such inhibitory nucleic acids to polymerase/reverse transcriptase suitable for use in the invention will be apparent to one of ordinary skill in the art or determined with no more than routine experimentation.

Inhibitory nucleic acid molecules of the invention may be synthesized by standard chemical oligonucleotide synthesis techniques (for example, phosphoramidite and others know in the art, see U.S. Patent No. 5,529,756). Alternatively, recombinant DNA techniques may be used to produce the inhibitory nucleic acids of the invention by cloning the nucleic acid molecule of interest into a vector, introducing the vector into the host cell, growing the host cell and isolating the inhibitory nucleic acid molecule of interest from the

host cell. Inhibitory nucleic acid molecules of the invention may also be obtained from commercial sources of custom oligonucleotides such as Life Technologies, Inc. or may be made enzymatically, for example, by using polymerases in nucleic acid synthesis or amplification reactions.

5            In some embodiments, the oligonucleotides of the present invention may be used for therapeutic purposes. In a preferred embodiment, the oligonucleotides of the present invention may be used to treat a subject (for example, a human or an animal) infected with a virus that requires reverse transcriptase activity to replicate. For therapeutic treatment, oligonucleotides  
10           may be administered as a pharmaceutically acceptable composition in which one or more oligonucleotides of the present invention may be mixed with one or more carriers, thickeners, diluents, buffers, preservatives, surface active agents, excipients and the like. Pharmaceutical compositions may also include one or more additional active ingredients such as antimicrobial agents,  
15           antiinflammatory agents, anesthetics, and the like in addition to oligonucleotides.

            The pharmaceutical compositions of the present invention may be administered by any route commonly used to administer pharmaceutical compositions. For example, administration may be done topically (including  
20           ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip or subcutaneous, intraperitoneal or intramuscular injection.

            Pharmaceutical compositions formulated for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays,  
25           liquids and powders. Any conventional pharmaceutical excipient, such as carriers, aqueous, powder or oily bases, thickeners and the like may be used.

            Pharmaceutical compositions formulated for oral administration may be in the form of one or more powders, granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Pharmaceutical  
30           compositions formulated for oral administration may additionally comprise

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thickeners, flavorings, diluents, emulsifiers, dispersing aids, binders or the like.

Pharmaceutical compositions formulated for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

The pharmaceutical compositions of the present invention may be administered in a therapeutically effective dose. A therapeutically effective dose is one which inhibits the replication of the virus within the host. It is not necessary that replication of the virus be entirely eliminated in order for a treatment to be therapeutically effective. Reduction of the rate of replication of the virus may be a therapeutic effect. One or more doses of the pharmaceutical compositions of the present invention may be administered one or more times daily for a period of treatment which may be a single administration or may be multiple administrations per day for a period of several days to several months or until a cure is effected or a diminution of disease state is achieved. Persons of ordinary skill can easily determine optimum dosages, dosing routes and the frequency at which doses should be administered.

**Polymerases.** Enzymes with polymerase activity to which the inhibitory nucleic acids of the present invention can bind or interact include any enzyme used in nucleic acid synthesis, amplification or sequencing reactions. Such polymerases include, but are not limited to, polymerases (DNA and RNA polymerases), and reverse transcriptases. DNA polymerases include, but are not limited to, *Thermus thermophilus* (Tth) DNA polymerase, *Thermus aquaticus* (Taq) DNA polymerase, *Thermotoga neopolitana* (Tne) DNA polymerase, *Thermotoga maritima* (Tma) DNA polymerase, *Thermococcus litoralis* (Tli or VENT™) DNA polymerase, *Pyrococcus furiosus* (Pfu) DNA polymerase, DEEPVENT™ DNA polymerase, *Pyrococcus woosii* (Pwo) DNA polymerase, *Pyrococcus* sp KOD2 (KOD) DNA polymerase, *Bacillus sterothermophilus* (Bst) DNA polymerase, *Bacillus caldophilus* (Bca) DNA polymerase, *Sulfolobus acidocaldarius* (Sac) DNA

polymerase, *Thermoplasma acidophilum* (Tad) DNA polymerase, *Thermus flavus* (Tfl/Tub) DNA polymerase, *Thermus ruber* (Tru) DNA polymerase, *Thermus brockianus* (DYNAZYME™) DNA polymerase, *Methanobacterium thermoautotrophicum* (Mth) DNA polymerase, mycobacterium DNA polymerase (Mtb, Mlep), *E. coli* pol I DNA polymerase, T5 DNA polymerase, T7 DNA polymerase, and generally pol I type DNA polymerases and mutants, variants and derivatives thereof. RNA polymerases such as T3, T5 and SP 6 and mutants, variants and derivatives thereof may also be used in accordance with the invention.

The nucleic acid polymerases used in the present invention may be mesophilic or thermophilic, and are preferably thermophilic. Preferred mesophilic DNA polymerases include pol I family of DNA polymerases (and their respective Klenow fragments) any of which may be isolated from organism such as *E. coli*, *H. influenzae*, *D. radiodurans*, *H. pylori*, *C. aurantiacus*, *R. Prowazekii*, *T. pallidum*, *Synechocysis sp.*, *B. subtilis*, *L. lactis*, *S. pneumoniae*, *M. tuberculosis*, *M. leprae*, *M. smegmatis*, Bacteriophage L5, *phi-C31*, T7, T3, T5, SP01, SP02, mitochondrial from *S. cerevisiae* MIP-1, and eukaryotic *C. elegans*, and *D. melanogaster* (Astatke, M. et al., 1998, *J. Mol. Biol.* 278, 147-165), pol III type DNA polymerase isolated from any sources, and mutants, derivatives or variants thereof, and the like. Preferred thermostable DNA polymerases that may be used in the methods and compositions of the invention include *Taq*, *Tne*, *Tma*, *Pfu*, *KOD*, *Tfl*, *Tth* Stoffel fragment, VENT™ and DEEPVENT™ DNA polymerases, and mutants, variants and derivatives thereof (U.S. Patent No. 5,436,149; U.S. Patent 4,889,818; U.S. Patent 4,965,188; U.S. Patent 5,079,352; U.S. Patent 5,614,365; U.S. Patent 5,374,553; U.S. Patent 5,270,179; U.S. Patent 5,047,342; U.S. Patent No. 5,512,462; WO 92/06188; WO 92/06200; WO 96/10640; WO 97/09451; Barnes, W.M. *Gene* 112:29-35 (1992); Lawyer, F.C., et al, *PCR Meth. Appl.* 2:275-287 (1993); Flaman, J.-M, et al., *Nucl. Acids Res.* 22(15):3259-3260 (1994)).



5 The enzymes having polymerase activity for use in the invention may be obtained commercially, for example from Life Technologies, Inc. (Rockville, Maryland), Perkin-Elmer (Branchburg, New Jersey), New England BioLabs (Beverly, Massachusetts) or Boehringer Mannheim Biochemicals (Indianapolis, Indiana). Enzymes having reverse transcriptase activity for use in the invention may be obtained commercially, for example, from Life Technologies, Inc. (Rockville, Maryland), Pharmacia (Piscataway, New Jersey), Sigma (Saint Louis, Missouri) or Boehringer Mannheim Biochemicals (Indianapolis, Indiana). Alternatively, polymerases or reverse transcriptases having polymerase activity may be isolated from their natural viral or bacterial sources according to standard procedures for isolating and purifying natural proteins that are well-known to one of ordinary skill in the art (see, e.g., Houts, G.E., et al., *J. Virol.* 29:517 (1979)). In addition, such polymerases/reverse transcriptases may be prepared by recombinant DNA techniques that are familiar to one of ordinary skill in the art (see, e.g., Kotewicz, M.L., et al., *Nucl. Acids Res.* 16:265 (1988); U.S. Patent No. 5,244,797; WO 98/47912; Soltis, D.A., and Skalka, A.M., *Proc. Natl. Acad. Sci. USA* 85:3372-3376 (1988)). Examples of enzymes having polymerase activity and reverse transcriptase activity may include any of those described in the present application.

***Methods of Nucleic Acid Synthesis, Amplification and Sequencing***

25 The inhibitory nucleic acids and compositions of the invention may be used in methods for the synthesis of nucleic acids. In particular, it has been discovered that the present inhibitory nucleic acids and compositions reduce nonspecific nucleic acid synthesis, particularly in amplification reactions such as the polymerase chain reaction (PCR). The present inhibitory nucleic acids and compositions may therefore be used in any method requiring the synthesis of nucleic acid molecules, such as DNA (including cDNA) and RNA molecules. Methods in which the inhibitory nucleic acids or compositions of



the invention may advantageously be used include, but are not limited to, nucleic acid synthesis methods and nucleic acid amplification methods (including "hot-start" synthesis or amplification) where the reaction is set up at a temperature where the inhibitory nucleic acid can competitively inhibit DNA synthesis or amplification and the synthesis or amplification reaction is initiated by increasing the temperature to reduce the competitive inhibition by the inhibitor of the polymerases thus allowing nucleic acid synthesis or amplification to take place.

Nucleic acid synthesis methods according to this aspect of the invention may comprise one or more steps. For example, the invention provides a method for synthesizing a nucleic acid molecule comprising (a) mixing a nucleic acid template with one or more primers and one or more inhibitory nucleic acids of the present invention (which may be the same or different) and one or more enzymes having polymerase or reverse transcriptase activity to form a mixture; (b) incubating the mixture under conditions sufficient to inhibit or prevent nucleic acid synthesis; and (c) incubating the mixture under conditions sufficient to make a first nucleic acid molecule complementary to all or a portion of the template. According to this aspect of the invention, the nucleic acid template may be a DNA molecule such as a cDNA molecule or library, or an RNA molecule such as a mRNA molecule or population of molecules. Conditions sufficient to allow synthesis such as pH, temperature, ionic strength, and incubation times may be optimized according to routine methods known to those skilled in the art.

In accordance with the invention, the input or template nucleic acid molecules or libraries may be prepared from populations of nucleic acid molecules obtained from natural sources, such as a variety of cells, tissues, organs or organisms. Cells that may be used as sources of nucleic acid molecules may be prokaryotic (bacterial cells, including those of species of the genera *Escherichia*, *Bacillus*, *Serratia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Chlamydia*, *Neisseria*, *Treponema*, *Mycoplasma*, *Borrelia*, *Legionella*, *Pseudomonas*, *Mycobacterium*, *Helicobacter*, *Erwinia*,







fragments), by methods described below and others that are standard in the art (see, e.g., U.S. Patent Nos. 4,962,022 and 5,498,523, which are directed to methods of DNA sequencing).

5 Nucleic acid sequencing methods according to the invention may comprise one or more steps. For example, the invention provides a method for sequencing a nucleic acid molecule comprising (a) mixing an enzyme with polymerase activity with one or more inhibitory nucleic acids of the present invention, a nucleic acid molecule to be sequenced, one or more primers, one or more nucleotides, and one or more terminating agents (such as a  
10 dideoxynucleotide) to form a mixture; (b) incubating the mixture under conditions sufficient to inhibit or prevent nucleic acid sequencing or synthesis; (c) incubating the mixture under conditions sufficient to synthesize a population of molecules complementary to all or a portion of the molecule to be sequenced; and (d) separating the population to determine the nucleotide  
15 sequence of all or a portion of the molecule to be sequenced.

Nucleic acid sequencing techniques which may employ the present inhibitory molecules or compositions include dideoxy sequencing methods such as those disclosed in U.S. Patent Nos. 4,962,022 and 5,498,523.

20 ***Vectors and Host Cells***

The present invention also relates to vectors which comprise an inhibitory nucleic acid molecule of the present invention. Further, the invention relates to host cells which contain the inhibitory nucleic acids of the  
25 invention and preferably to host cells comprising recombinant vectors containing such nucleic acids, and to methods for the production of the nucleic acids of the invention using these vectors and host cells. Nucleic acid synthesis and amplification products produced by the methods of the invention may also be cloned into vectors and host cells in accordance with the invention  
30 to facilitate production of such nucleic acid molecules or proteins encoded by such nucleic acid molecules.



cell growth. Appropriate culture media and conditions for the above-described host cells and vectors are well-known in the art. Following its production in the host cells, the nucleic acid or protein of interest may be isolated by several techniques. To liberate the nucleic acid or protein of interest from the host cells, the cells are preferably lysed or ruptured. This lysis may be accomplished by contacting the cells with a hypotonic solution, by treatment with a cell wall-disrupting enzyme such as lysozyme, by sonication, by treatment with high pressure, or by a combination of the above methods. Other methods of bacterial cell disruption and lysis that are known to one of ordinary skill may also be used.

Following disruption, the nucleic acid or proteins may be separated from the cellular debris by any technique suitable for separation of particles in complex mixtures. The nucleic acids or proteins may then be purified by well known isolation techniques. Suitable techniques for purification include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, electrophoresis, immunoabsorption, CsCl centrifugation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, liquid chromatography (LC), high performance LC (HPLC), fast performance LC (FPLC), hydroxylapatite chromatography and lectin chromatography.

### ***Kits***

The present invention also provides kits for use in the synthesis, amplification or sequencing of nucleic acid molecules. Kits according to this aspect of the invention may comprise one or more containers, such as vials, tubes, ampules, bottles and the like, which may comprise one or more of the inhibitory nucleic acids and/or compositions of the invention.

The kits of the invention may comprise one or more of the following components: (i) one or more nucleic acids or compositions of the invention;

(ii) one or more polymerases and/or reverse transcriptases, (iii) one or more suitable buffers or buffering salts; (iv) one or more nucleotides; and (v) one or more primers.

5 It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included  
10 herewith for purposes of illustration only and are not intended to be limiting of the invention.

### *Example 1*

#### *Nucleic Acid Inhibitors*

15 Nucleic acid inhibitors were synthesized by Life Technologies, Inc. and were HPLC or PAGE purified.

##### *Nucleic Acid Inhibitor A (34-mer)*

20 5'CCCAATATGGACCGGTCGAAAGACCGGTCCATAT<sup>3'</sup> (SEQ ID NO:1)

##### *Nucleic Acid Inhibitor B (55-mer)*

25 5'CCATGCAGGTAGCCGATGAACTGGTCGAAAGACCAGTTCATCGGC  
TACCTGCATG<sup>3'</sup> (SEQ ID NO:2)

At ambient temperature the above sequences form a hairpin-like structure (Antao and Tinoco, 1992, supra), see structures below.

30 In some embodiments, the 3'-terminus of Inhibitor A may be capped at the 3' terminus with a dideoxythymine triphosphate using a Klenow fragment mutant (F762Y) of DNA polymerase I (*Escherichia coli*) or T7 DNA polymerase (Tabor, S. and Richardson, C.C., 1995, *Proc. Natl. Acad. Sci. USA* 92, 6339-6343). The 3'-OH terminus of the oligonucleotide was extended





## ***Example 2***

### ***Inhibition of Polymerase with Inhibitor***

5 The time course of the activity of *Tne* DNA polymerase was  
qualitatively determined using a 34/60-mer primer/template substrate at 3  
different temperatures. Figure 1 represents results from these experiments.  
For each temperature, polymerase activity was measured in the absence (Panel  
A) and presence (Panel B) of inhibitor A. Aliquots of the reaction mixture  
were taken at various time points and separated on an agarose gel. The five  
10 lanes, of each panel, from left to right at 15 sec, 30 sec, 1 min, 2 min and 5  
min, time points that have elapsed before the reactions were quenched. P and  
C denote the primer position and control lane, respectively.

As can be seen in Figure 1, the potency of the inhibition of the  
polymerase reaction catalyzed by *Tne* is significantly reduced as the  
15 temperature is increased.

## ***Example 3***

### ***Amplification of a Target DNA Sequence from Plasmid DNA Source***

20 A 2.7 Kb target DNA sequence delivered from pUC19 plasmid was  
amplified using 5 different dilutions of the template. The target was amplified  
by *Tne* DNA polymerases. Two different concentration of *Tne* polymerase (85  
nM (e.g., 1 unit) and 42.5 nM (i.e., 0.5 units) and the *Tne* complexed to the  
inhibitor nucleic acid (using a 150-fold excess inhibitor B over polymerase)  
25 were used at each amplification condition. The results are shown in Figure 2.  
The concentration of the target DNA in lanes 1, 2, 3, 4 and 5 denote 100 pg,  
20 pg, 2 pg, 0.2 pg and 0.02 pg, respectively.

Results indicate that the sensitivity of *Tne* was greatly improved by the  
addition of the inhibitor, and relative purity of the target molecule was  
30 immensely enhanced.

#### ***Example 4***

##### ***Amplification of a Target DNA Sequence from Genomic DNA Source***

5 A 1 Kb, 3 Kb and 5 Kb target DNA sequences were amplified by *Tne* (85 nM (e.g., 1 unit) and *Taq* (1 unit) DNA polymerases as represented in panels A, B and C of Figure 3, respectively. The four lanes of each panel represented as a, b, c and d are *Tne* (+ 125-fold excess inhibitor A (ddT capped)), *Tne* (+50-fold excess inhibitor A (ddT capped)), *Tne* (no inhibitor) and *Taq* (no inhibitor), respectively.

10 Results indicate that the sensitivity of *Tne* was greatly improved by the addition of the inhibitor and relative purity of the target molecule was immensely improved for each target condition.

#### ***Example 5***

##### ***Amplification of a 5 and 15 Kb Target DNA Sequence by *Tne* DNA Polymerase***

15 The five panels A, B, C, D and E of Figure 4 represent reaction conditions: control *Tne* (no inhibitor), *Tne* (+50-fold excess inhibitor B), *Tne* (+150-fold excess inhibitor B), *Tne* (+300-fold excess inhibitor B), *Tne* (+750-fold excess inhibitor B), respectively. The two lanes in each panel represented as A and B are for amplification of target size of 5 and 15 Kb. The final concentration of the *Tne* DNA polymerase in each reaction was 8.5 nM (e.g., 0.1 unit).

25 As can be seen in Figure 4, the sensitivity of *Tne* was greatly improved by the addition of this inhibitor, and relative purity of the target molecule was immensely improved as the concentration of the inhibitor was optimized. As shown in panels D and E, 15 Kb product can only be amplified by *Tne* DNA polymerase, under *Taq* PCR conditions (Perkin-Elmer), in the presence of the  
30 inhibitor nucleic acid.

### ***Example 6***

#### ***Amplification of a 3 Kb Target DNA Sequence (Human Genomic Source) by 1 Unit Taq***

##### DNA Polymerase

5           The three panels A, B, and C of Figure 5 represent reaction conditions.  
For A and B, the same primer sequences were used. In A, the PCR mix was  
incubated at 94°C for 1 min and was set on ice to force mis-priming. All PCR  
reaction were set for 30 min at 25°C so as to increase non-specific DNA  
synthesis. Each condition: lane A (*Taq* control), B (*Taq* + 126 nM inhibitor),  
10       C (*Taq* + 32 nM inhibitor) and D (*Taq* + 64 nM inhibitor).

Results show that the specificity of *Taq* was greatly improved by the  
addition of the inhibitor in each case producing significant reduction in the  
non-specific DNA synthesis and enhanced amount of the target sequence  
product.

15

### ***Example 7***

#### ***Inhibition of RT Using the Oligonucleotides of the Present Invention***

20           The DNA polymerase activity of ThermoScript™ I RNase deficient  
mutant reverse transcriptase (RT) (available from Life Technologies, Inc.) was  
determined at ambient temperature, 37°C and 55°C in the presence and  
absence of the oligonucleotide inhibitor molecules. The sequences and  
secondary structures of the oligonucleotide inhibitors are shown below. The  
polymerase activity of the RT was determined under steady state kinetic  
25       conditions using olig(dG)<sub>15</sub>/polyrC as the primer/template substrate. This  
assay has been described by Polesky *et al.* (1990), and was used with minor  
modification.





Hairpin Structure of Nucleic Acid Inhibitor G

G

A GGTATA A T G A G A G TATATA<sup>-3'</sup>

A CCAUAUCAUUAUUAUUAUGUAAUUA<sup>-5'</sup>

5 G (SEQ ID NO:7)

Nucleic Acid Inhibitor H (synthesized by Life Technologies, Inc.)

A 50 mer DNA/RNA hybrid nucleic acid, RNA bases are underlined.

5' AAUUA AUGUAUAUAUUAUUAUUA ACUAUACCGAAAATATATAATGATG  
10 ATATAG<sup>3'</sup> (SEQ ID NO:8)

The relative polymerase activities of ThermoScript™ I in the absence and presence of nucleic acid inhibitors at ambient temperature (~22°C), 37°C and 55°C was determined. The polymerization reaction was initiated by the addition of RT or RT/inhibitor (5 µL) to a solution of the primer/template in the presence of dGTP (spiked with dGT<sup>32</sup>P) and MgCl<sub>2</sub>, final reaction volume of 50 µL. The mixture was incubated at the reaction temperature, and samples (5 µL) were removed at 1 min (22°C) and 15 sec (37°C and 55°C) intervals and were added into 50 µL of 25 mM EDTA. A portion of the quenched solution was applied to DE-81 filters. Following washes to remove unincorporated dGTP, the filters were counted in scintillation vials containing EconoFluor-2 (Packard). The apparent rate of the reaction was derived from the rate plot (com plotted against time interval). The reaction concentration of the oligo(dG)<sub>15</sub>/polyrC was 800 nM in primer, dGTP was 100 µM and MgCl<sub>2</sub> and KCl were 10 mM and 50 mM, respectively. For each reaction condition the concentration of the reverse transcriptase was maintained at 12 nM whereas the concentration of each of the oligonucleotide inhibitor was 540 nM.

The relative activity of the polymerization reaction catalyzed by ThermoScript™ at ambient temperature, 37°C and 55°C in the presence and absence of the inhibitors are shown in Figure 6A and 6B. The activities in the

absence of the oligonucleotide inhibitors (free ThermoScript™ I) was normalized to 1 for measurements at each temperature. The RT activity in the presence of an inhibitor was correlated to the activity of ThermoScript™ at each temperature and the relative normalized activities are presented as a bar graph and are shown in Figures 6A and 6B. For each set of reaction condition, the three bars from left to right denote reactions performed at ambient temperature, 37°C and 55°C, respectively. TS, TS-D, TS-E, and TS-H in Figure 6A denote polymerase reaction initiated by ThermoScript™ I, ThermoScript™ I-nucleic acid inhibitor D complex, ThermoScript™ I-nucleic acid inhibitor E complex and ThermoScript™ I-nucleic acid inhibitor H complex, respectively. The efficiency of inhibition of the RT activity is dependent to the temperature which indicates that the level of inhibition of RT by the nucleic acid inhibitors is dependent to the melting temperature of the nucleic acid.

***Nucleic Acid Inhibitor D***

Under experimental conditions described above, complexing ThermoScript™ I with about 50-fold excess of this nucleic acid prior to initiating the polymerization reaction inhibited the RT activity by about 85-90% at each of the reaction temperatures. The relative similarity of the level of inhibition is indicative of the stability of the hairpin structure of this nucleic acid inhibitor in the temperature range that was assayed for RT activity.

***Nucleic Acid Inhibitor E***

Under the experimental conditions, complexing ThermoScript™ I with about 50-fold excess of this nucleic acid prior to initiating the polymerization reaction inhibited the RT activity by about 90% at ambient temperature and 37°C but the level of inhibition was 45% at 55°C. The significant reduction in the level of inhibition at 55°C suggests that the hairpin structure was







***Nucleic Acid Inhibitor G***

Under the experimental conditions, complexing ThermoScript™ I with about 50-fold excess of this nucleic acid prior to initiating the polymerization reaction inhibited the RT activity by about 80% at ambient temperature, 55% at 37°C and 30% at 55°C. The decrease in the level of inhibition in correlation to the increase of the reaction temperature is indicative of the destabilization of the hairpin structure due to the three mis-matches.

***Example 8***

***Inhibition of Reverse Transcriptase Activity Within a Cell***

The oligonucleotides of the present invention may be used to inhibit the activity of a reverse transcriptase enzyme with a cell. Oligonucleotides for use inside a cell may optionally be modified to render them resistant to one or more nuclease enzymes that may be present in a cell. For example, a derivative of the nucleic acid inhibitor may be synthesized with one or more of the following modifications: 1) one or more of the ribose groups on the RNA portion of the oligonucleotide may be alkylated, for example, methylated, preferably at the 2'-OH to produce a 2'-O methyl; 2) one or more of the internucleotide linkages of the oligonucleotide, for example, in the DNA portion of the nucleic acid, may contain a modified linkage, for example, a phosphorothioate linkage; 3) the 3' terminus of the oligonucleotide may be capped so as to be non-extendable, for example, with a phosphate, phosphorothioate or a dideoxynucleotide or other modification of the 3'-hydroxyl so as to make it not extendable. Other modifications that increase the level of resistance to cellular RNase activity and/or reduce the efficiency of DNA degradation by other cellular factors are known to those skilled in the art and may be incorporated into the design of the oligonucleotides of the present invention. In addition to rendering the oligonucleotides resistant to one or more cellular degradation factors, phosphorothioate reduces the possibility of

homologous recombination into the host chromosome should a given inhibitor contain a region homologous to one on the host chromosome.

Olligonucleotides may be assayed to determine if they have an inhibitory effect on reverse transcriptase activity in a cell. Cells to be treated with the oligonucleotides of the invention, for example NIH3T3 cells may be transfected with the nucleic acid (Life Technologies, Inc.) using any method known to those skilled in the art. In some preferred embodiments, the oligonucleotides of the invention may be introduced into the cells using lipid mediated transfection (see, for example, U.S. Patent Nos. 5,334,761; 5,674,908; 5,627,159; 5,736,392; 5,279,833 and published international application WO 94/27345 all of which are specifically incorporated herein by reference).

Following transfection of the cells, a virus expressing a reverse transcriptase (for example, Moloney Murine Leukemia V, M-MLV) may be added so as to efficiently infect cells with the virus and provide a source of reverse transcriptase activity. (Jolicoeur, P. and Rassart, E., 1980). An aliquot of the cells will be centrifuged and lysed at time intervals in order to assay for reverse transcriptase activity. Comparing the level of RT activity derived from cells that are transfected with a nucleic acid inhibitor and those that are not transfected, the efficacy of inhibition of viral proliferation in the cell can be determined.

### ***Example 9***

#### ***Inhibition of Taq polymerase Using Phosphorothioate Substituted Oligonucleotides***

In some preferred embodiments, one or more phosphorothioate residues may be incorporated into the oligonucleotides of the present invention. Those skilled in the art will appreciate that oligonucleotides incorporating such internucleotide linkages may be more resistant to nuclease activities that may be present in a reaction mixture or within a cell.



HPHH2 is a phosphorothioate hairpin oligonucleotide with a 3 nucleotide loop, a melting temperature of the duplex region of 67°C and a  $\Delta G = -18.10$  kcal/mol of formation of the duplex.



(SEQ ID NO:10)

HPHH3 is a phosphorothioate hairpin oligonucleotide with a 5 nucleotide loop and a melting temperature of the duplex region of 70°C and a  $\Delta G = -18.90$  kcal/mol of formation of the duplex.



(SEQ ID NO:11)

HPHH4 is a phosphorothioate oligonucleotide with a 4 nucleotide loop and a melting temperature of 65°C of the duplex and a  $\Delta G = -13.5$  kcal/mol of formation of the duplex.



(SEQ ID NO:12)

The bases involved in formation of the stem structure are indicated by a vertical line. When the oligonucleotide was modified at the 3'-terminal with a 3 carbon spacer group ending in a hydroxyl, the designation Sspa3 was added to the name of oligonucleotide.

With reference to Figure 7, amplification reactions to produce a 1.6 kb (A), a 2 kb (B) and a 2.6 kb (C) fragment of the NF2 gene. In each panel, lane a is the amplification using *Taq* polymerase alone, lane b is the amplification reaction in the presence of inhibitor HPHH4Sspa3 at a molar ratio of 1.2:1 inhibitor: polymerase and lane c is the amplification using Platinum *Taq*.

The template was 200 ng of genomic DNA. A comparison of lane b to lanes a and c in each panel shows that the presence of the inhibitor improves the amount of full length product and reduces the amount of shorter products under these reaction conditions.

5           As shown in Figure 8, the activity of *Taq* polymerase in a nucleotide incorporation assay was determined at three temperatures (25°C, 55°C and 72°C) in the presence of two different concentrations of inhibitor HPHH4Sspa3 (molar ratios of 2:1 and 7.5:1 inhibitor:polymerase). At each temperature, the solid black bar is the *Taq* polymerase alone, the striped bar is  
10 *Taq* polymerase plus inhibitor at a 2:1 ratio of inhibitor to polymerase and the solid white bar is *Taq* plus inhibitor at a 7.5:1 ratio of inhibitor:polymerase. The incorporation was assayed in a PCR reaction mixture incubated at the indicated temperature in the presence of alpha-[<sup>32</sup>P]-dCTP. After 30 minutes, the reactions were stopped by the addition of EDTA and an aliquot of each  
15 reaction was spotted onto GF/C filters. The filters were washed with TCA and counted. The activity was normalized to the amount of activity of the *Taq* polymerase at the same temperature.

          At 25°C and a 2:1 ratio, *Taq* activity was reduced to approximately 60% of the un-inhibited polymerase while a 7.5:1 ratio reduced activity by  
20 approximately 90%. At 55°C (a typical annealing temperature) inhibition was still observed, approximately a 20% and 60% reduction in activity at 2:1 and 7.5:1 respectively. At 72°C (a typical extension temperature) inhibition was nearly eliminated at a 2.5:1 ratio and was approximately 50% at a 7.5:1 ratio. These data indicate that the inhibition is temperature dependent with more  
25 inhibition observed at lower temperatures (*i. e.*, when the oligonucleotide is in a hairpin structure) and less at a higher temperature.

          The concentration dependence of the inhibition of *Taq* polymerase by inhibitor HPHHSspa3 was studied at 37°C and the results are shown in Figure 9. The incorporation assay described above was used. These results indicate  
30 that the inhibition is dose dependent with a slight (20%) inhibition seen at a molar ration of 0.5:1 inhibitor: polymerase up to a nearly complete inhibition

